The metabolism of possible cytokinin precursors supplied to excised maize roots

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Excised maize roots were grown, *in vitro*, on nutrient media supplied with labelled mevalonic acid, isopentenylpyrophosphate or glycine. After 35 days incubation the roots were separated from the agar medium. Both the roots and the respective culture media were extracted for cytokinin-like activity. Biological activity was detected in both roots and media in all cases. Approximately all radioactivity detected was associated with the applied compound in the case of mevalonic acid and isopentenylpyrophosphate. Where roots were supplied with labelled glycine small amounts of labelled adenine and adenosine were detected in the root extracts. In no case was radioactivity found to be associated with free cytokinins.

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Geïsoleerde mieliewortels is *in vitro* op voedingsmedia wat radioaktiewe mevalonsuur, isopentenielpirofosfaat of glisien bevat het, gekweek. Na 35 dae is die wortels van die media geskei. Beide die wortels en die verskillende media is vir sitokinien-aktiwiteit getoets. Biologiese aktiwiteit is in alle gevalle in die wortels en media gevind. In die geval waar mevalonsuur en isopentenielpirofosfaat aangewend is, was die meeste van die waargenome radioaktiwiteit met hierdie twee verbindings geassosieer. Waar die wortels van glisien voorsien is, is klein hoeveelhede gemerkte adenien en adenosien in die wortelekstrakte gevind. In geen geval is radioaktiwiteit geassosieer met vry sitokiniene gevind nie.

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Introduction

For some years there has been a certain amount of controversy regarding the mechanism of biosynthesis of free cytokinins. Two major mechanisms have been suggested. The first involves biosynthesis via tRNA; since cytokinin active bases occur in tRNA, free cytokinins may be formed by cytokinin containing RNA. However, the extremely low rate of turnover of the tRNA and the differences in the chemical nature of the free and tRNA-bound cytokinins suggest that this is not the major source of free cytokinins (Stuchbury *et al.* 1979). The second, and probably more widely accepted mechanism, is that of *de novo* biosynthesis. There is increasing evidence that plant tissues contain an enzyme capable of the direct synthesis of free cytokinins (Holtz & Klämbt 1978; Nishinari & Syóno 1980a & b). The enzyme system was originally isolated from maize and is said to catalyse the transfer of the isoprene unit of isopentenylpyrophosphate to adenosine (Holtz & Klämbt 1978).

Roots have been proposed as sites of synthesis of cytokinins (Weiss & Vaadia 1965; Engelbrecht 1972; Van Staden & Smith 1978; Forsyth & Van Staden 1981). Although the evidence is largely circumstantial it was decided to utilize excised maize roots to investigate cytokinin biosynthesis in the present study.

Adenine and adenosine have been suggested as precursors of zeatin and its derivatives, that is, the free cytokinins (Burrows 1978; Chen & Petschow 1978; Stuchbury *et al.* 1979; Nishinari & Syóno 1980a). Thus if roots are sites of synthesis and adenine or adenosine can act as precursors it should be possible to supply excised roots with radioactive adenine and recover labelled free cytokinins. In a previous study (Van Staden & Forsyth 1984) no such incorporation was found. It is possible that a shoot-derived precursor such as isopentenylpyrophosphate was absent in this system. This compound, which is the likely side chain precursor (Nishinari & Syóno 1980b), is reported as being synthesized in chlorophyllous tissue (Grumbach & Forn 1980). Thus, in the present investigation excised maize roots were cultured in the presence of the labelled, suggested precursors mevalonic acid, isopentenylpyrophosphate or glycine in an attempt to recover labelled free cytokinins.

Materials and Methods

Kernels of *Zea mays* L. cv. Hickory King were surface sterilized for 15 min with concentrated commercial bleach, washed with sterile distilled water and allowed to germinate on a 0,7% agar medium at 25 °C. After 10 days incubation the sterile roots were excised and transferred aseptically to 50 cm^3 Erlenmeyer flasks containing 30 cm^3 half strength

Miller's (1965) medium from which cytokinin and auxin were omitted. The medium contained 3% sucrose and was solidified with 0,7% agar. Radiochemicals were purchased from the Radiochemical Centre, Amersham. DL — (2-^{14}C) mevalonic acid (specific activity 0,8 GBq mmol^{-1}), (1-^{14}C) isopentenylpyrophosphate (specific activity 2 GBq mmol^{-1}) and (1-^{14}C) glycine (specific activity 2,1 GBq mmol^{-1}) were added to the respective flasks prior to autoclaving. The roots were incubated at 25 °C for 35 days whereafter they were separated from the agar medium. Both the roots and the agar were separately frozen and subsequently, individually analysed for cytokinin activity. A Dowex 50 cation exchange resin was used to purify the samples (Van Staden 1976) which had been extracted overnight in 80% ethanol at 5 °C. The purified extracts were taken to dryness *in vacuo* at 40 °C and the residue dissolved in 3 cm^3 80% ethanol. These extracts were applied directly to Sephadex LH-20 columns and fractionated using 10% methanol as eluant (Hutton & Van Staden 1981). Fractions of 40 cm^3 each were collected. A small aliquot (2 cm^3) from each fraction was transferred to a scintillation vial, 10 cm^3 Ready-solv E.P. scintillation cocktail added and the radioactivity assessed using a Beckman LS 3800 scintillation counter. The remainder of the extract was used for the determination of cytokinin-like activity, using the soya bean callus bioassay (Miller 1965) and, where necessary, HPLC techniques.

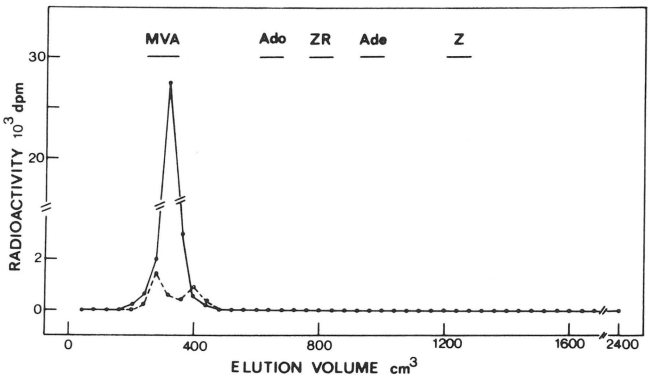

Figure 1 Radioactivity recovered from excised maize roots (○– – –○) and the medium on which they were grown (●——●). Cultures were supplied with ^{14}C-mevalonic acid. Extracts were purified using Dowex 50 cation exchange resin, concentrated and then applied directly to Sephadex LH-20 columns using 10% methanol as eluant. MVA — mevalonic acid; Ado — adenosine; ZR — ribosylzeatin; Ade — adenine; Z — zeatin.

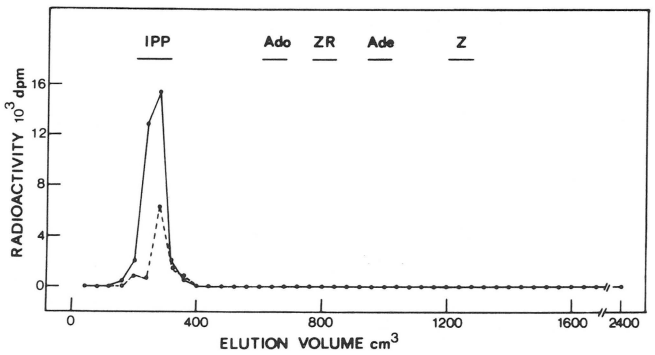

Figure 2 Radioactivity recovered from excised maize roots (○– – –○) and the medium on which they were grown (●——●). Cultures were supplied with ^{14}C-isopentenylpyrophosphate. Extracts were purified using Dowex 50 cation exchange resin, concentrated and then applied directly to Sephadex LH-20 columns using 10% methanol as eluant. IPP — isopentenylpyrophosphate, other abbreviations as in Figure 1.

Before use, a sample of each of the three compounds used as radioactive additives was tested for radiochemical purity by Sephadex LH-20 fractionation following Dowex 50 purification. In each case a single radioactive peak was obtained. The elution volume of each peak is indicated on the appropriate figure (Figures 1 – 4).

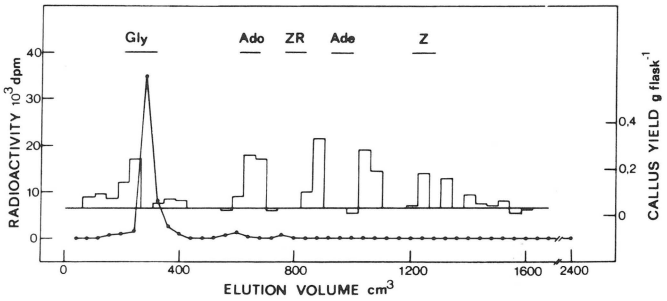

Figure 3 Radioactivity recovered (●——●) and biological activity (histogram) of the medium on which excised maize roots were grown. Cultures were supplied with ^{14}C-glycine. The extract was purified using Dowex 50 cation exchange resin, concentrated and then applied directly to a Sephadex LH-20 column using 10% methanol as eluant. Gly — glycine, other abbreviations as in Figure 1.

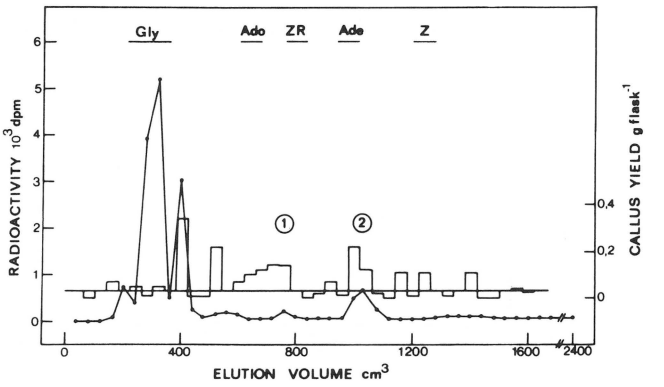

Figure 4 Radioactivity recovered (●——●) and biological activity (histogram) of excised maize roots supplied with ^{14}C-glycine. The extract was purified using Dowex 50 cation exchange resin, concentrated and then applied directly to a Sephadex LH-20 column using 10% methanol as eluant. Gly — glycine, other abbreviations as in Figure 1.

Results

Figures 1 – 4 indicate that radioactivity was recovered from both root and agar extracts in all cases. The total radioactivity recovered was consistently higher in the agar fractions but, nevertheless, recovery of radioactivity from the root extracts indicates that, in all cases, the applied compound was taken up by the excised roots.

Where mevalonic acid and isopentenylpyrophosphate were applied approximately 100% of the recovered radioactivity in both root and agar extracts was associated with the respective applied compound (Figures 1 & 2, respectively). The same was true of the radioactivity recovered from the agar extract which had been supplied with glycine (Figure 3). However, analysis of the maize roots supplied with glycine revealed three polar radioactive peaks, the largest of which co-eluted with authentic glycine and the other two very much smaller and more non-polar (Figure 4). Radioactivity associated with the three polar peaks (elution volume 160 – 480 cm^3) accounted for approximately 73% of the total recovered radioactivity. Of the two small, non-polar peaks, peak 1

(elution volume 720 – 800 cm^3) represented 1,7% of the total radioactivity and 6,3% of the radioactivity not associated with polar peaks. Peak 2 (elution volume 960 – 1120 cm^3) represented 7% of the total recovered radioactivity and 26% of the radioactivity not associated with polar peaks.

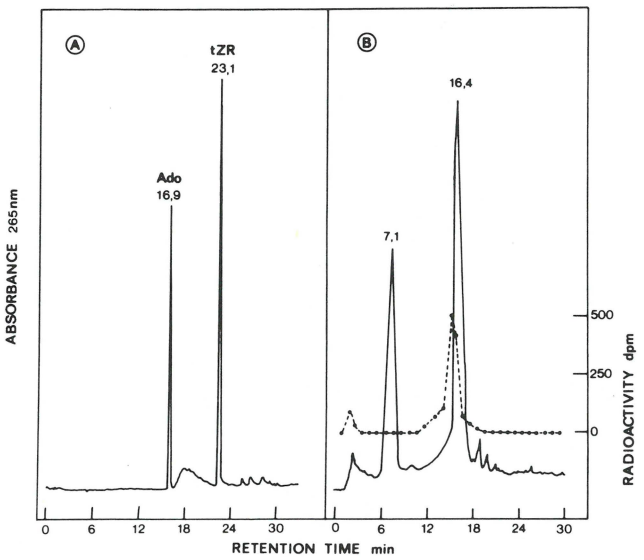

Figure 5 Separation of authentic purine derivatives (A) and radioactive peak 1 (see Figure 4) (B) obtained from excised maize roots by reversed-phase HPLC. Column Hypersil 5 ODS, 250 × 4 mm I.D.; flow rate 1,5 cm^3 min^{-1}. Mobile phase, water to 4% acetonitrile over 10 min, then to 30% acetonitrile over 20 min. Absorbance was recorded with a Varian variable wavelength monitor at 265 nm which was fitted with an 8 mm^3 flow through cell. Separation was achieved using a Varian 5000 Liquid Chromatograph. (●– –●) — Radioactivity; (———) — UV-absorbance; Ado — adenosine; tZR — transribosylzeatin.

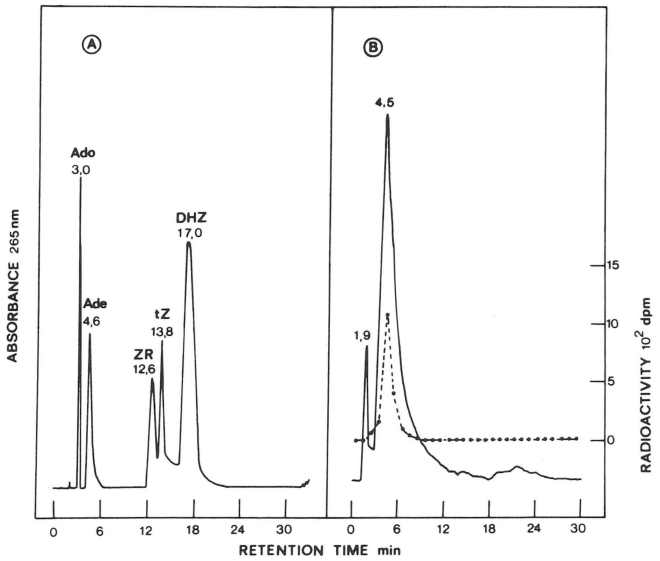

Figure 6 Separation of authentic purine derivatives (A) and radioactive peak 2 (see Figure 4) (B) obtained from excised maize roots by reversed-phase HPLC. Column Hypersil 5 ODS, 250 × 4 mm I.D.; flow rate 1,5 cm^3 min^{-1}. Mobile phase, 10% acetonitrile, 90% water for 10 min, to 14% acetonitrile, 86% water over 5 min, maintained for 5 min, then to 30% acetonitrile, 70% water over 5 min, maintained for 5 min, to 100% methanol over 5 min. Absorbance was recorded with a Varian variable wavelength monitor at 265 nm which was fitted with an 8 mm^3 flow through cell. Separation was achieved using a Varian 5000 Liquid Chromatograph. (●– –●) — Radioactivity; (———) — UV-absorbance; Ado — adenosine; Ade — adenine; ZR — ribosylzeatin; tZ — trans zeatin; DHZ — dihydrozeatin.

Peaks 1 and 2 therefore appeared to be products of glycine metabolism which could represent 'free' cytokinin activity and thus were further investigated using HPLC techniques. The results of these investigations are shown in Figures 5 & 6.

The nature of Peak 1 was investigated using a solvent system of water to 4% acetonitrile over 10 min, then to 30% acetonitrile over 20 min. The column used was a Hypersil 5 ODS. Using this system Peak 1 had a retention time of 16,4 min and co-eluted with authentic adenosine (Figure 5). The solvent system used to investigate the nature of Peak 2 was 10% acetonitrile, 90% water for 10 min, to 14% acetonitrile, 86% water over 5 min, maintained for 5 min, then to 30% acetonitrile over 5 min, maintained for 5 min, to 100% methanol over 5 min. The column used was a Hypersil 5 ODS. Using this system Peak 2 had a retention time of 4,5 min and co-eluted with authentic adenine (Figure 6).

Discussion

The literature relating to the mechanism of free cytokinin biosynthesis consistently points to adenine and/or adenosine being the precursor/s for the biosynthesis of free cytokinins (Peterson & Miller 1976; Burrows 1978; Chen & Petschow 1978; Stuchbury *et al.* 1979; Nishinari & Syóno 1980a). Since roots are considered to be sites of cytokinin biosynthesis it was surprising that no incorporation of labelled adenine or adenosine was found in a previous study (Van Staden & Forsyth 1984). It is postulated that the isopentenyl group is enzymatically attached to adenosine and the resultant N^6-(isopentenyl) adenosine is, again enzymatically, converted to zeatin (Nishinari & Syóno 1980b). It was therefore thought that since the maize roots lacked a shoot system it was possible that the side chain precursor, thought to be synthesized in chlorophyllous tissue (Grumbach & Forn 1980), may have been absent in this case. The adenine pool in any plant cell is likely to be a relatively large one because of the central role of adenine in metabolism (Wareing *et al.* 1976), thus, in the present study the addition of mevalonic acid and isopentenyl-pyrophosphate was investigated. The results shown are entirely negative with respect to free cytokinin biosynthesis.

Excised roots were then cultured with labelled glycine as it was thought that this may be a more readily utilizable precursor for adenine biosynthesis. The results show a certain amount of metabolism of glycine and the recovery of low levels of compounds with similar retention times to those of adenosine and adenine.

Previous reports of the incorporation of adenine or adenosine into free cytokinins come from studies using shoot systems (Chen & Petschow 1978), cell suspension cultures (Nishinari & Syóno 1980a), cell-free extracts (Nishinari & Syóno 1980b) or cytokinin autonomous callus (Burrows 1978; Stuchbury *et al.* 1979). Accepting that roots are possible sites of synthesis of cytokinins, it would not be unreasonable to expect to find that the full complement of precursors and enzymes is synthesized or available within these organs. If these assumptions are valid it is difficult to explain why no incorporation of labelled compounds into free cytokinins was found in the present study. Since biological activity was detected in both root and agar fractions in all cases (but only depicted in Figures 3 & 4 for the sake of clarity) some cytokinin biosynthesis must have taken place. It is possible that once the roots were excised from the kernels biosynthesis was immediately reduced to an extremely low rate, insufficient to metabolize the applied compounds.

Another possibility is that the applied compounds followed a degradative rather than a biosynthetic route. Both these

pathways have been shown to be operative during germination of *Phaseolus mungo* seeds (Ashihara 1983). Adenine and adenosine are said to be salvaged preferentially for nucleotide and nucleic acid synthesis (Ashihara 1983), implying a biosynthetic route via the nucleotides. However, in the present investigation, nucleotide fractions yielded no radioactive metabolites. Ashihara (1983) suggests that the pathway followed by applied compounds, whether biosynthetic or degradative, may depend on the relative activities of the various enzyme systems involved.

Acknowledgement

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